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THE TOXIN OF BACILLUS WELCHII. I

TOXIN PRODUCTION BY VARIOUS STRAINS

PAUL H. DEKRUIF, THEODORE W. ADAMS,
AND
PAUL M. IRELAND

From the Hygienic Laboratory, University of Michigan, Ann Arbor.

Bull and Pritchett[1] were the first to demonstrate that B. welchii produces a soluble toxin. Metchnikoff,[2] Korentchevsky,[3] and others had obtained toxic substances, but these did not have the characteristics generally attributed to exotoxins. They were comparatively heat stabile and did not produce antitoxins on injection into the animal body. The first named investigators, by using a special medium and by incubating the cultures for a relatively short time were able to produce a toxin corresponding to those of diphtheria and tetanus, in that it was destroyed by heat of 60-70 C. and caused antitoxin production when injected into the animal body.

We have thought it desirable to test the toxin-producing ability of various strains of Bacillus welchii, and to determine the neutralizing power of one antitoxin against the toxins from these various strains. We have also looked into the question of toxin production by methods other than those used by Bull and Pritchett.

THE COMPARATIVE TOXICOGENIC POWER OF TEN STRAINS OF BACILLUS WELCHII

The organisms used in these tests, with the exception of B. perfringens, and Strain 617d, were isolated in this laboratory. B. perfringens is the so-called "souche toxique" of Weinberg and Séguin, Strain 617d was received from Dr. Bull. This strain, which was the most powerfully toxicogenic of any of those used by Bull and Pritchett, was used to control the toxicity of the other strains.

Strains 1, 5, 7, 8, 9, 10, 11 and 17 were isolated from the stools of healthy individuals. A thick suspension of fecal material in 0.85% NaCl was made, filtration being resorted to for the removal of fecal masses; 0.5 c.c. of this suspension was placed in a tube of freshly boiled and cooled litmus milk. The mixture was at once heated to 80 C. for 60 minutes. The cutures were then incubated, and if the characteristic stormy fermentation developed, dilution tubes with 0.2% glucose agar were made according to the method of Veillon. The typical gas colonies growing in the depths of the medium were fished and transplanted into litmus milk.

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[1] Jour. Exper. Med., 1917, 26, p. 119.

[2] Ann. de l'Inst. Pasteur, 1908, 22, p. 929.

[3] Ibid., 1909, 23, p. 91.

Great confusion exists in regard to the criteria necessary to place an unknown organism in the species of B. welchii. It would be well then to sum up the more important characteristics of the strains just mentioned. All of the organisms are nonmotile, gram-positive rods with slightly rounded ends. They grow singly or in pairs, and when in the latter grouping exhibit a characteristic V formation. In the exudates of animals dying from infections, especially when these are produced by intraperitoneal inoculation, occasional long chains may be met with. The organisms all produce gas violently in dextrose agar and broth and give rise to the typical "stormy fermentation" in litmus milk. They do not form spores in carbohydrate medium, but do so readily in coagulated egg-white broth. The odor of butyric acid is very strong in milk and glucose-broth cultures. None of the cultures exhibit a putrefactive odor. Coagulated egg-white is not attacked even after several days at incubator temperature. The bacilli grown in milk and broth are very sensitive to the large amount of acid produced in such medium and survive for a few days at room or incubator temperature. Dead cultures may exhibit perfectly preserved forms, but the organisms, besides failing to grow on transplantation, will be found to have become gram-negative.

The Method of Toxin Production

The method used in the comparative tests was that of Bull and Pritchett[1] with a single modification. The medium was placed in 100 c.c. Erlenmeyer flasks, rather than in test tubes, as recommended by these authors. Fifty c.c. of 0.1% glucose broth were placed in each flask. Veal and Witte peptone were used in the preparation of the broth, and the reaction was adjusted to + 0.5. Portions of sterile rabbit muscle, removed under aseptic conditions, were placed in the broth. The amount of muscle used was approximately equal to that of the liquid portion of the medium. Each flask was then inoculated with 1 c.c. of an 18-hour milk culture, covered with a layer of sterile paraffin oil, placed in a vacuum jar and exhausted to 28 inches. After such exhaustion the flasks were incubated at 37 C. for 18 hours. The medium was then removed from under the oil by a bulb pipet and centrifuged to remove the bacilli and tissue débris. The supernatant fluid was filtered by suction through Berkefeld N filters and the filtrate was tested for sterility. The toxicity of the filtrates was then tested by intramuscular injection into guinea-pigs of 300 gm. weight.

All of the toxin tests recorded in Table 1 were made on the same day, from the same batch of medium. The toxin of Strain 617d was used as a control in these tests. It will be noted that the toxin produced by this organism was 3 times more powerful in Table 1 than in Table 2. In many different trials the potency of its toxin has varied between 0.05 and 0.5 c.c. The average variation, however, is not as great as this. In the majority of cases, lethal doses of from 0.1 to 0.3 c.c. were obtained.

The abdominal surfaces of the guinea-pigs under test were carefully shaved. The injections were made into the muscles of the thigh. The standard for a lethal dose of the toxin was the smallest amount of toxin necessary to kill a 300 gm. guinea-pig in 48 hours. The standard used by Bull and Pritchett is 24 hours for a 350 gm. pigeon, but it has been found that while the guinea-pig does not react as rapidly as the pigeon there is no great difference in susceptibility to the toxin.

TABLE 1

The Comparative Toxin Production of Strains 8, 9, 10 and 617d

Strain	C.c. of Toxin	Result	Minimal Lethal Dose
8	1.0	+ 12 hours	0.5
	0.5	+ 19 hours	
	0.3	Severe necrosis	
	0.1	Moderate necrosis	
9	1.0	+ 10 hours	
	0.5	+ 12 hours	
	0.3	+ 10 hours	
	0.2	+ 21 hours	0.2
	0.1	Slight necrosis	
	0.05	0	
10	1.0	+ 12 hours	
	0.5	+ 13 hours, 30 minutes	
	0.3	+ 21 hours, 30 minutes	
	0.2	+ 22 hours	0.2
	0.1	Severe necrosis	
617d	1.0	+ 12 hours	
	0.5	+ 12 hours	
	0.3	+ 10 hours	
	0.2	+ 11 hours	
	0.1	+ 21 hours, 30 minutes	0.1
	0.05	Slight necrosis	

The findings at necropsy were similar to those recorded by Bull and Pritchett. The muscles at the site of inoculation are necrotic and there is a marked outpouring of gelatinous exudate over the subcutaneous abdominal surface. In animals that survive small doses of toxin extensive necrosis of not only the injected leg, but also the abdominal surface occurs. This is followed by sloughing and secondary infection.

Another series of tests was made with Strains 1, 5, 7, 11, 17, and B. perfringens. Graded doses were employed the same as in the experiments given in Table 1, but for the sake of brevity only the minimum lethal dose for each strain is given in Table 2.

It will be noted that in this case Strain 617d, which was used as a control, did not develop the most toxic filtrate. Strain 11 seems to be the most potent in this series. It is also worthy of note that the B. perfringens is a very poor toxin producer as compared with some of the strains isolated by us. Repeated tests of the toxicogenic power of this organism have been made, and in all cases the minimum lethal dose has varied between 1.0 and 2.0 c.c.

These results indicate that toxin production is a property common to many different strains of B. welchii. Not a single one of the 10 strains tested by us has failed to produce at least to some extent, a soluble toxic product. It is possible that subsequent work will show this to be a property so constant as to be of use in determination of the identity of the organism.

TABLE 2

THE COMPARATIVE TOXICITY OF STRAINS 1, 5, 7, 11, 17, B. PERFRINGENS

Strain	Minimal Lethal Dose
1	0.5
5	0.75
7	1.0
11	0.1
17	2.0
B. perfringens	1.0
617d	0.3

It was of interest to ascertain whether all these toxins would be subject to neutralization by one and the same antitoxin. The antitoxin used in these experiments was kindly furnished us by Dr. Bull. One two-hundred and fiftieth of a c.c. protected a 350 gm. pigeon against 1 lethal dose. This amount, according to Bull, is designated as 1 unit of antitoxin. We did not determine whether the neutralizing power of this antitoxin was greater for guinea-pigs than for pigeons, but regarded the previously mentioned amount of antitoxin as 1 unit.

The tests given in Table 3 were made by mixing multiples of a guinea-pig lethal dose of toxin with corresponding units of antitoxin, allowing the mixtures to stand at the temperature of the room for 30 minutes, and at the end of this time injecting the mixture intramuscularly into guinea-pigs of 300 gm. weight. Controls of a similar number of lethal doses of toxin mixed with a corresponding amount of normal horse serum were made in every case.

The results given in Table 3 indicate that the antitoxin used neutralizes the toxins from every strain tested, and go far to show that the filtrates from the various strains are of a common nature. It will be noted that in many instances some signs of intoxication were present in the animals which had received toxin-antitoxin mixtures. These were in every case of a transient nature and were confined to slight or moderate edemas which disappeared and were not followed by any distinct necrosis.

TABLE 3
Neutralization of Various Toxins by a Single Antitoxin

Number Strain	C.c. of Toxin	Number of Minimal Lethal Doses	C.c. Antitoxin, 1:50	C.c. of Normal Horse Serum 1:50	Result
1	1.5	3	0.6	...	Nil
	1.5	3	...	0.6	+ 26 hours
5	1.5	2	0.4	...	Marked edema
	1.5	2	...	0.4	+ 15 hours
7	3.0	3	0.6	...	Nil
	3.0	3	...	0.6	+ 14 hours
8	2.5	5	1.0	...	Slight edema
	2.5	5	...	1.0	+ 16 hours
9	1.0	5	1.0	...	Very slight edema
	1.0	5	...	1.0	+ 17 hours
10	0.5	5	1.0	...	Slight edema
	0.5	5	...	1.0	+ 20 hours
11	0.3	3	0.6	...	Very slight edema
	0.3	3	...	0.6	25 hr., 30 min.
B. perfringens	2.0	2	0.4	...	Nil
	2.0	2	...	0.4	+ 16 hr., 30 min.
617d	0.5	5	1.0	...	Marked edema
	0.5	5	...	1.0	+ 15 hours

It would be desirable to test carefully the binding relations of this toxin and its antitoxin. While Bull and Pritchett have shown that this relation follows roughly the law of multiple proportions, it would seem extremely probable that the phenomenon of Bordet-Danysz would be encountered here, as in the case of tetanus and diphtheria toxins and their antitoxins. Plans for such determinations are at present under way.

VARIOUS METHODS OF PREPARING THE TOXIN

As antitoxin bids fair to have widespread use, especially during the war, consequently it would seem desirable to produce the toxin by as simple a method as possible. We have accordingly studied toxin production under various conditions.

Tests were first made to determine the amount of sterile muscle necessary for the production of a potent toxin. Fifty c.c. of 0.1% glucose, +0.5 reaction, veal broth were placed in each of three Erlenmeyer flasks, A, B, and C. Sterile rabbit muscle was placed in Flask A in such amount as approximately to double the volume. Flask B received enough muscle to increase the volume by one fifth only. Flask C received no muscle. All 3 of the flasks were now inoculated with an 18-hour milk culture of Strain 617d, and after covering the surfaces with oil and exhausting in the usual manner, were incubated for 18 hours. Following this the cultures were filtered and centrifugated. The filtration was performed through a Berkefeld N filter. The injections were made intramuscularly. The results are given in Table 4.

TABLE 4
THE RELATION OF QUANTITY OF STERILE MUSCLE TO TOXIN PRODUCTION

Set	Guinea-Pig		C.c. of Toxin	Result
	Number	Weight		
A	1	300	1.0	+ 8 hours
	2	300	0.5	+ 8 hours
	3	300	0.3	+26 hours
	4	300	0.1	Severe necrosis; recovered
B	5	300	1.0	+ 10 hours
	6	300	0.5	+ 10 hours
	7	300	0.3	+ 22 hours, 30 minutes
	8	300	0.1	Severe necrosis; recovered
C	9	300	2.0	+ 10 hours
	10	300	1.5	+ 22 hours
	11	300	1.0	Edema and moderate necrosis
	12	300	0.5	Slight necrosis

It will be observed from Table 4 that it makes no material difference whether large or small amounts of muscle are used. The lethal dose for A and B is the same. On the other hand, the toxicity of the culture without muscle was about a fifth as great as that of A and B.

We now attempted to determine the importance of paraffin oil in toxin production. It was found that very potent toxins can be obtained without the addition of this substance. In one case parallel tests were made of the toxicity of filtrates from 2 flasks, 1 of which had not been covered with oil. The toxicity of this filtrate reached the high value of 0.05 c.c. The filtrate from the oil-covered flask showed a lethal value of 0.1 c.c. This variation is of course too minute to be of importance, but the experiment indicated that the layer of oil was unnecessary in toxin production. Since this test was made we have never used the oil.

Other methods of anaerobiosis were tried with good results. Absorption of oxygen with pyrogallol proved to be efficacious, though no more so than the exhaustion method. Finally it was found that neither of these procedures was necessary, but that simple preliminary boiling of the medium before inoculation was all that was required for excellent growth of the organisms and resulting toxin production.

THE PRODUCTION OF HIGH TITER TOXIN WITHOUT STERILE MUSCLE

It was remarked previously that some toxin could be produced without the use of sterile muscle, the aseptic removal of which from rabbits introduces a factor of considerable complication into the method of toxin production, especially when this is to be conducted on an extensive scale. It would be highly desirable, however, to obtain a maximum yield of toxin and our attempts were directed toward this end.

TABLE 5

The Toxicity of Veal-Mash-Broth Filtrates (Strain 11)

	Number	C.c. of Toxin	Minimum Lethal Dose	Result
A Veal Mash-Broth (1:1)	1	1.0	...	+ 14 hours
	2	0.5	...	+ 15 hours
	3	0.3	...	+ 18 hours
	4	0.2	...	+ 40 hours
	5	0.1	0.1	+ 48 hours
B Veal Mash-Broth (1:2)	1	1.0	...	+ 12 hours
	2	0.5	...	+ 14 hours
	3	0.3	0.3	+ 21 hours
	4	0.2	...	Severe edema; recovered
	5	0.1	...	Slight edema; recovered
C Broth, No Veal Mash (Control)	1	1.5	...	+ 12 hours
	2	1.0	...	+ 12 hours
	3	0.5	...	+ 15 hours
	4	0.4	0.4	+ 36 hours
	5	0.3	...	Severe edema; recovered
	6	0.2	...	Severe edema: recovered
	7	0.1	...	Slight edema

Many workers experienced in the growing of anaerobic bacteria have found that suspensions of autoclaved minced beef or veal furnish an excellent medium for these organisms. At the suggestion of Dr. Novy we attempted the production of toxin by the substitution of autoclaved veal for the sterile rabbit muscle.

Strain 11 was used in this experiment, which was conducted as follows: A 0.1% glucose, + 0.5 reaction, veal broth was prepared. One hundred c.c. of this medium were placed in each of 3 flasks. To Flask A were added 100 gm. of finely chopped veal, to Flask B 50 gm., while no veal was added to the control Flask C. All 3 of the flasks were now autoclaved at 110 C. for 30 minutes. At once after cooling each flask was inoculated with 1.0 c.c. of an 18-hour milk culture of B. welchii, Strain 11. The cultures were then incubated, without exhaustion or covering with paraffin oil, for 18 hours. A heavy growth of characteristic organisms was observed in all of the flasks, although that in the flask without meat was not as rich as that in Flasks A and B. The fluid portion of the medium was now withdrawn by means of a bulb pipet, centrifugated at 8,000 revolutions per minute and filtered through Berkefeld N filters. The toxicity of the 3 filtrates was tested by intramuscular injection into guinea-pigs of 300 gm. weight. The result of the experiment is shown in Table 5.

It will be observed then, that this method furnishes an excellent substitute for that in which sterile rabbit muscle is used. It reduces considerably the complication of technic, and when a good toxin producing strain is used furnishes a toxin concentration as great as that obtained by the method of Bull and Pritchett (Table 2).

SUMMARY AND CONCLUSIONS

The toxicogenic power of 10 strains of B. welchii has been tested with the result that all the strains have been found to produce toxin in greater or less degree.

An antitoxin produced by the injection of toxin from a single strain is capable of neutralizing all of the toxins. This fact furnishes strong evidence of the common nature of the toxic products of the various strains.

Various methods of toxin production have been tested. It has been determined that a layer of sterile paraffin oil is not necessary for securing growth or toxicity. Methods of removing oxygen, such as exhaustion or absorption are superfluous. It is necessary only to boil the medium before use.

A considerable concentration of toxin may be secured without the addition of sterile muscle. The lowest minimum lèthal dose secured by this method has been up to the present, 0.4 c.c. The method of choice which combines simplicity with a comparatively high concentration of toxin, is the veal mash-broth cultivation we described. In this method the complicating factor of aseptic muscle is avoided.